# Native Chromatin Immunoprecipitation (N-ChIP)

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## 1. The preparation of native chromatin from cultured human cells

1.1. Cultured cells (e.g. HL-60 or lymphoblastoids) are grown to a density of approximately 1 x $10^6$ cells/ml until they are in log phase.

1.2. Harvest cells: centrifuge samples (7,000 g, 10 min, 4°C) and wash the cell pellet 3 x ice cold PBS (Phosphate Buffered Saline).

*It is essential that 5 mM Na butyrate is present in all solutions throughout chromatin isolation when using antibodies to acetylated histones to prevent deacetylation.*

1.3. Resuspend cell pellet in TBS (Tris buffered saline) at 2 x $10^7$ cells/ml and add an equal volume of 1.0% v/v Tween 40 in TBS. Add PMSF to a final concentration of 0.5 mM. Leave stirring gently on ice for 1hr (Transfer the suspension into a 50ml tube with a small magnetic bar or flea; place the tube in ice on top of a magnetic stirrer).

1.4. Transfer cell lysate to an all-glass homogeniser and homogenise 7 ml aliquots with seven strokes using an 'A' or 'tight' pestle. Check that nuclei have been released by phase-contrast microscopy; intact cells should have the central dark region of the nucleus surrounded by a halo, which is the less dense cytoplasm.

*You may have to increase or decrease this homogenisation step to maximise the yield of nuclei depending on cell line.*

1.5. Centrifuge samples (10,000 g, 20 min, 4°C).

1.6. Resuspend nuclei pellet in 25% [w/v] sucrose/TBS at 4x$10^6$ nuclei / ml and underlay with 0.5 vol of 50% [w/v] sucrose / TBS; centrifuge the samples (14,000 g, 25 min, 4°C).

1.7. Discard supernatant and wash nuclei pellet in 5 ml 25% [w/v] sucrose/TBS; centrifuge samples (14,000 x g, 25 min, 4°C).

1.8. Resuspend nuclei pellet in 5ml digestion buffer and check absorbance ratios at 260 nm and 280 nm for a diluted sample of the nuclei suspension; calculate the approximate DNA concentration from the A260 reading (the ratio of A260/A280 should be about 1.1). Centrifuge samples (10,000 rpm, 10 min, 4°C) and resuspend the nuclei pellet at 0.5mg/ml in 1.7 ml Eppendorf tube(s).

## 2. Micrococcal nuclease digestion

Normally we add 50 U micrococcal nuclease per 0.5 mg DNA, in a reaction volume of 1.0 ml. This is usually provided as a powder; dissolve the micrococcal nuclease in $dH_20$ to the required concentration and store as small aliquots at -20°C. Aliquots may be re-frozen and re-used once. This step needs to be carefully controlled, especially in the initial preparations.

*High concentrations of micrococcal nuclease may over-digest the chromatin, leading to sub-nucleosomal particles. You should aim to obtain a long/medium oligonucleosome ladder. If pure mononucleosome preparations are required carry out a linear sucrose gradient (5-20%), this will increase resolution.*

2.1. Perform microccal nuclease digestions at 37°C for 5 min.

2.2. Stop reaction by addition of 0.2 M EDTA to a final concentration of 5 mM.

2.3. Place all samples on ice for 5 min; centrifuge samples (8,000 g, 5 min).

2.4. Remove and keep the first S/N (this is called the S1 fraction; total vol 1.0 ml); store overnight at 4°C.

2.5. Resuspend the pellet in 1.0 ml Lysis buffer and dialyse overnight against 2 litres of the same buffer.

2.6. After overnight dialysis centrifuge samples (500 g, 10 min, 4°C).

2.7. Remove and keep the supernatant (called the S2 fraction; total vol about 1.2 ml after dialysis); store at 4°C.

2.8. Resuspend insoluble pelleted material in 200 µl lysis buffer (called the P fraction).

## 3. Analysis of soluble chromatin fractions

3.1. Check A260/A280 in all samples; the ratios for S1, S2 and P fractions are approximately 1.7, 1.5 and 1.3 respectively.

3.2. Analyze all samples by 1.2% agarose gel electrophoresis.

*Do not place ethidium bromide in the agarose gel or the electrophoresis buffer, because of the presence of SDS (see below).*

3.3. Preparation of samples: x µl (total of 5 µg) chromatin fraction (S1, S2 and P) y µl dH2O (x+y = 25 µl) 3 µl 1% [w/v] SDS (final conc 0.1%) 2 µl gel loading buffer, containing bromophenol blue

3.4. Stain the gel with 0.5 µg/ml ethidium bromide after the run has finished.

## 4. Immunoprecipitation

4.1. 100-200 µg unfixed chromatin + 100-200 µl affinity purified antibody (50-100 µg Ig) and the final volume made up to 1.0 ml with incubation buffer. A negative control, with no added antibody, also needs to be set up to test for any nonspecific binding of the chromatin to the protein A Sepharose.

4.2. Incubate overnight at 4°C on a slow rotating turntable. Add 200 µl 50% v/v protein A Sepharose; use a siliconized pipette with the tip cut off to make this step easier. Incubate for 3 hr at room temperature on a fast rotating turntable. (Make sure that the Sepharose is in a suspension at all times).

4.3. Centrifuge samples (3,000 g, 10 min, 4°C), remove and keep the S/N; this is the unbound (or “U”) fraction.

4.4. Resuspend the Sepharose pellet in 1ml buffer A and layer onto 9ml of the same buffer using a siliconised pasteur pipette and siliconized 15 ml tube.

4.5. Centrifuge samples (10,000 g, 10 min, 4°C), discard the S/N and wash the Sepharose sequentially in 10 ml buffer B and buffer C.

4.6. Finally, resuspend the Sepharose in 1 ml buffer C and transfer back to siliconized Eppendorfs.

4.7. Centrifuge samples (3,000 g, 10 min, 4°C) and resuspend the sepharose pellet in 250 µl 1.0% SDS / incubation buffer and incubate for 15 min at RT on a fast turntable. (Ensure that the Sepharose is thoroughly resuspended at all times).

4.8. Centrifuge the samples (3,000 g, 10 min, 4°C) and remove and keep S/N; this is the bound (or “B”) fraction.

4.9. Wash the sepharose in 250 µl 1.0% SDS / incubation buffer and centrifuge immediately (3,000 g, 10 min, 4°C). Remove the S/N and pool with the previous bound fraction from the previous step.

## 5. DNA Isolation

Add 500 µl incubation buffer to each bound fraction, to reduce the SDS concentration to 0.5% SDS.

Unbound and bound fractions then treated as follows:

5.1. Add 0.33 vol (330 µl) phenol/chloroform; vortex and spin (13,000 rpm, 10 min, microcentrifuge). Keep the organic phase and interface; this is used to isolate immunoprecipitated proteins (see below).

5.2. Transfer the aqueous supernatant to an equal volume (1.0 ml) of phenol/chloroform; vortex and spin (13,000 rpm, 10 min, microcentrifuge)

5.3. Transfer supernatant to an equal volume (1.0 ml) of chloroform; vortex and spin (13,000 rpm, 10 min, microcentrifuge)

5.4. Transfer S/N to a clean centrifuge tube and add 0.1 vol (100 µl) 4 M LiCl, 50 µg glycogen (Molecular biology grade, dissolved in $dH_20$ at 2 mg/ml) as a carrier and 4 vol of ethanol. Vortex thoroughly and leave at -20°C overnight.

5.5. Centrifuge samples (13,000 g, 15 min) to precipitate the DNA.

5.6. Wash the pellet with 70% ethanol and redissolve the DNA in 250 µl TE buffer.

5.7. Store samples at -20°C or proceed with detection method (PCR, microarray, etc).

5.8. PCR is used to quantify DNA levels of specific loci. This is analyzed semi-quantitatively (analyses of PCR end-product by agarose gel) using primers which can be designed using the URL below.

http://biotools.umassmed.edu/bioapps/primer3_www.cgi

Alternatively, DNA levels are quantitatively measured by real-time PCR. Primers and probes are often designed using software provided with the real-time PCR apparatus.

## 6. Protein Isolation

6.1. To the first phenol/chloroform phase (see DNA isolation; step1) add 5 µl of a 1 mg/ml solution of BSA (to be used as a carrier), 0.01 vol (4 µl) 10 M $H_2SO_4$ and 12 vol of acetone.

6.2. After precipitation at -20°C wash the protein pellets once in acidified acetone (1:6 100 mM $H_2SO_4$:acetone) and 3 times in dry acetone. Proteins can be analyzed by SDS-PAGE.

## Solutions

**10 x TBS**
0.1 M Tris-HCl (pH 7.5)
1.5 M NaCl
30 mM $CaCl_2$
20 mM $MgCl_2$
50 mM Na butyrate (pH 8.0)

**Digestion buffer**
0.32 M sucrose
50 mM Tris-HCl (pH 7.5)
4 mM $MgCl_2$
1 mM $CaCl_2$
0.1 mM PMSF
5 mM Na butyrate

**Lysis buffer**
1.0 mM Tris-HCl (pH7.4)
0.2 mM $Na_2EDTA$
0.2 mM PMSF
5 mM Na butyrate

**Incubation buffer**
50 mM NaCl
20 mM Tris-HCL (pH 7.5)
20 mM Na butyrate
5 mM $Na_2EDTA$
0.1 mM PMSF

**Buffer A**
50 mM Tris-HCl, (pH 7.5)
10 mM EDTA
5 mM Na butyrate
50 mM NaCl

**Buffer B**
50 mM Tris-HCL (pH 7.5)
10 mM EDTA
5 mM Na butyrate
100 mM NaCl

**Buffer C**
50 mM Tris-HCL (pH 7.5)
10 mM EDTA
5 mM Na butyrate
150 mM NaCl

**Protein A Sepharose**
Pre-swell protein A Sepharose overnight in **buffer A** at 4°C. Centrifuge (10,000 x g, 10 min) and resuspend pellet in approximately an equal volume (50% v/v) of **buffer A.**

*(Adapted from protocols used by Laura O'Neill and Prof. Bryan Turner. University of Birmingham).*